DEVELOPMENT OF AN ANTI-MUC1 ANTIBODY-DRUG CONJUGATE WITH SPECIFICITY TO TARGET CANCER

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by **Kelsey Coyle**, titled ***Development of an Anti-Muc1 Antibody-Drug Conjugate with Specificity to Target Cancer*** and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.

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ABSTRACT

Ever since Paul Ehrlich, Nobel Prize winner and the founder of chemotherapy, postulated that "magic bullets" can be created and used to fight human disease, scientist have been inspired to develop precise and tailored drugs to target cancer [1]. This vision came true with the development of a therapeutic that forms highly specific associations with targeted antigens by Kohler and Milstein in the form of monoclonal antibodies (mAb) [2]. Huge advances have been made in the past decade with mAb therapy of cancer to treat many common malignancies with over 206 mAbs studied in clinical trials from 1980 to 2005 [2,3]. Despite these advances, cancer is still affecting and killing numerous lives every day.

The monoclonal antibody therapy for cancer is deemed tailored because it specifically targets antigens expressed on cancer cells. Mucin 1 is a transmembrane mucin that is overexpressed in a number of metastatic epithelial cancers [4,5]. Its expression correlates with an aggressive form of disease, poor response to therapy, and poor survival [4]. The differences between the antigen in tumor and normal cells in terms of biochemical features, cell distribution, and function [5] provide an opportunity to use antibodies to specifically target and attack Muc1 positive tumor cells. Anti-Muc1 antibodies alone, however, have not proven to be sufficiently cytotoxic to kill tumor cells.

Depending on type and stage, current cancer treatments include surgery, radiation, chemotherapy, and immunotherapy. The goal of chemotherapy is to kill the fast-growing cancer cells. However, the drugs are often toxic so that they kill they normal cells in the body, leading to critical side effects. In this project, we aim to combine the high specificity properties of anti-Muc1 antibodies with the highly potent chemotherapeutic agent in order to identify a 'magic bullet' immune-based

treatment that produces improved efficacy and leads to the specific destruction of tumor-associated Muc1 cancer cells.

INTRODUCTION

Problem: Cancer

According to the American Cancer Society, lung cancer is the second most common cancer in both men and women with about 13% of all new cancers being of the lung [6]. Not only is the cancer prevalent, but it is one of the deadliest, accounting for 25% of cancer deaths [7]. Furthermore, lung cancer is the leading cause of cancer death by far with more people dying of it than of colon, breast, and prostate combined [6]. Currently, the amount of resources available to treat lung cancer nowhere near correlates with the impact of the disease. Given this, we are in desperate need to find better screening tests and treatment options.

Non-small cell lung cancer (NSCLC), specifically, accounts for 84% of all lung cancer diagnoses, making it the most common type of lung cancer [8]. Current treatment is based on the progression of the cancer. Treatments can include: surgery, radiation, chemotherapy, and immunotherapy. Outcomes do improve dramatically if the cancer is caught before metastasis because surgery is often a very effective form of treatment. However, most patients with NSCLC are often at an advanced stage by the time of diagnosis. Overall prognosis is very poor, with the five-year survival rate of only 23% [8]. More work needs to be done in order to address prevention, early detection, and treatment of lung cancer.

As stated above, Mucin 1, a transmembrane member of the mucin family, is a protein overexpressed and altered in many epithelial cancers. Its expression is often associated with aggressive metastasis and poor prognosis [5]. Muc1 expression correlates with cancer progression and is associated with the following cancers: lung, breast, ovarian, prostate, gastrointestinal, liver, and pancreas. Table 1 illustrates that Mucin 1 expression is associated with many different cancers. In this project, we will focus primarily on NSCLC, which exhibits 99% Muc1 expression (see Table 1).

MUCIN 1 IS OVEREXPRESSED IN MANY CANCERS

TUMOR TYPE	MUC1 EXPRESSION	# TISSUES EXAMINED	SOURCE
Nasopharyngeal	100%	38	Zhong XY, et al. 1993
Non-small cell lung	99%	231	EMD Serono, Inc. Data on file
Breast	91%	1447	Rakha EA, et al. 2005
Renal cell carcinoma	84%	133	Langner C, et al. 2004
Ovarian	83%	63	Chauhan SC, et al. 2006
Squamous cell carcinoma of head and neck	82%	29	Croce MV, et al. 2001
Colorectal	81%	243	Baldus SE, et al. 2002
Pancreatic	81%	53	Qu CF, et al. 2004
Prostate	79%	89	DeNardo SJ, et al. 2005
Gastric	77%	136	Ultsunomiya T. et al. 1998
Mesothelioma	75%	20	Saad RS, et al. 2005
Multiple myeloma	73%	26	Cloosen S, et al. 2006
Esophageal	32%	53	Kijima H, et al. 2001

Table 1: Mucin 1 is Overexpressed in Many Cancers. The overexpression of Mucin 1 protein is seen in a variety of different epithelial cancers (adapted from table made by Kerry Jordan at EMD Serono).

Muc1 Relationship

Under normal conditions, mucins are large, heavily glycosylated proteins that are protectors and controllers of the cell surface. They are often found in particularly harsh environments and are expressed by epithelial cells of various organs, including the stomach, intestinal tract, liver, pancreas, gall bladder, kidney, eyes, esophagus, prostate, uterus, and lung [9,10]. In healthy tissues, mucins aid in protecting the underlying epithelia at sites of respiration, digestion, and excretion [5,11]. These sites experience rapid changes in environment in regard to pH, ionic concentrations, oxygenation, and toxin exposure [5]. With these variable conditions, mucins play a key role in maintaining homeostasis and promoting cell survival [9] (see Figure 1).

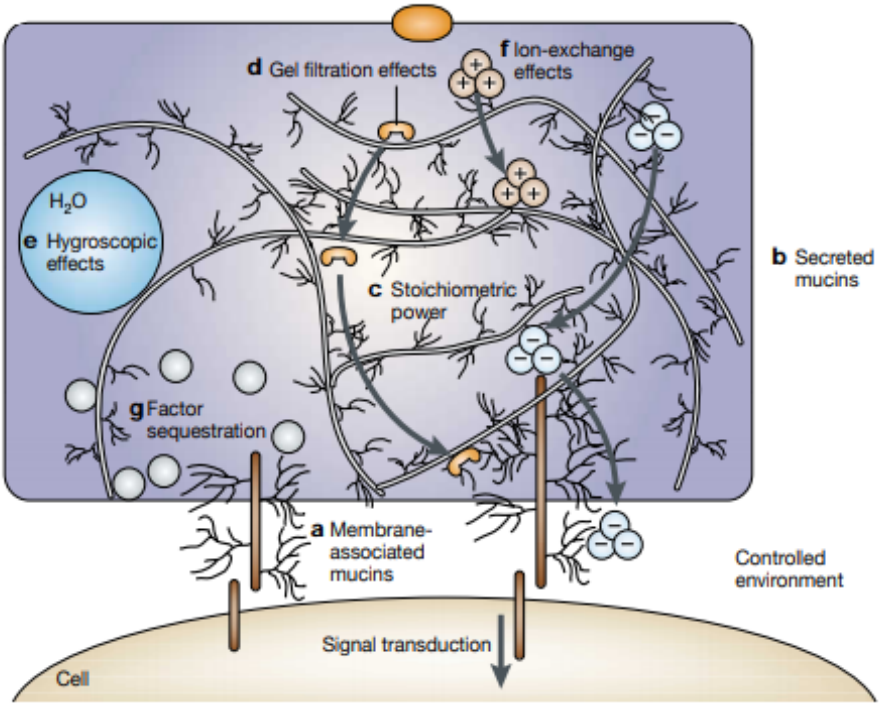

Figure 1: Roles of Mucins. Mucins play a variety of roles in controlling the environment both in and around the cell, including: signal transduction, molecular sensing, protection, filtration, and maintenance. Reproduced from Swanson et al (2004) with permission.

Mucin 1, specifically, is a heavily O-glycosylated transmembrane protein that acts to protect that underlying epithelia. The long, negatively charged sugar branches create a physical barrier that imparts an anti-adhesive property [5]. Mucin 1 consists of two domains: the long N-terminal subunit (Muc1-N) and the short C-terminal subunit (Muc1-C) [5] (see Figure 2). These subunits remain associated through hydrogen bonds extracellularly and create a heterodimer at the sea urchin sperm protein enterokinase and agrin (SEA) domain, which is a glycine-serine proteolytic cleavage site [12]. The Muc1-N subunit contains the variable number tandem repeat (VNTR) region, which encodes a variable number of sequences encoding 20 amino acids, rich with serines and threonines, that are extensively O-glycosylated under normal conditions [5] (see Figure 2).

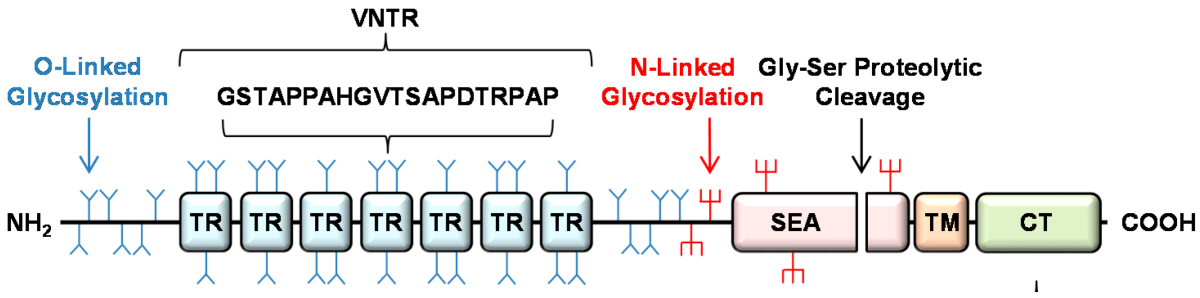

Figure 2: Mucin 1 Structure. Mucin 1 consists of Muc1-N and Muc1-C domains. The VNTR region is rich in serines and threonines that become extensively O-glycosylated under normal conditions. Reproduced from Kato et al (2017) with permission.

There are many differences between normally expressed Muc1 and tumor-associated Muc1 in terms of biochemical features and cell distribution. In tumor cells, Muc1 becomes hypoglycosylated; this unmasks the peptide core, which destabilizes the cell surface and makes the protein vulnerable to proteolytic cleavage and clathrin-mediated endocytosis [5]. Furthermore, the loss of polarity of cancer cells causes the overexpressed Muc1 to be redistributed over the

entire cell surface [5] (see Figure 3). Another difference between tumor associated Muc1 and that found on noncancerous cells is the changes in interaction between Muc1-C and Muc1-N. When Muc1 becomes hypoglycosylated, it allows Muc1-N to be cleaved and released from the cell surface [13]. All of these alterations in Muc1 expression, features, distribution, and function have been shown to impact disease progression through various mechanisms as described below.

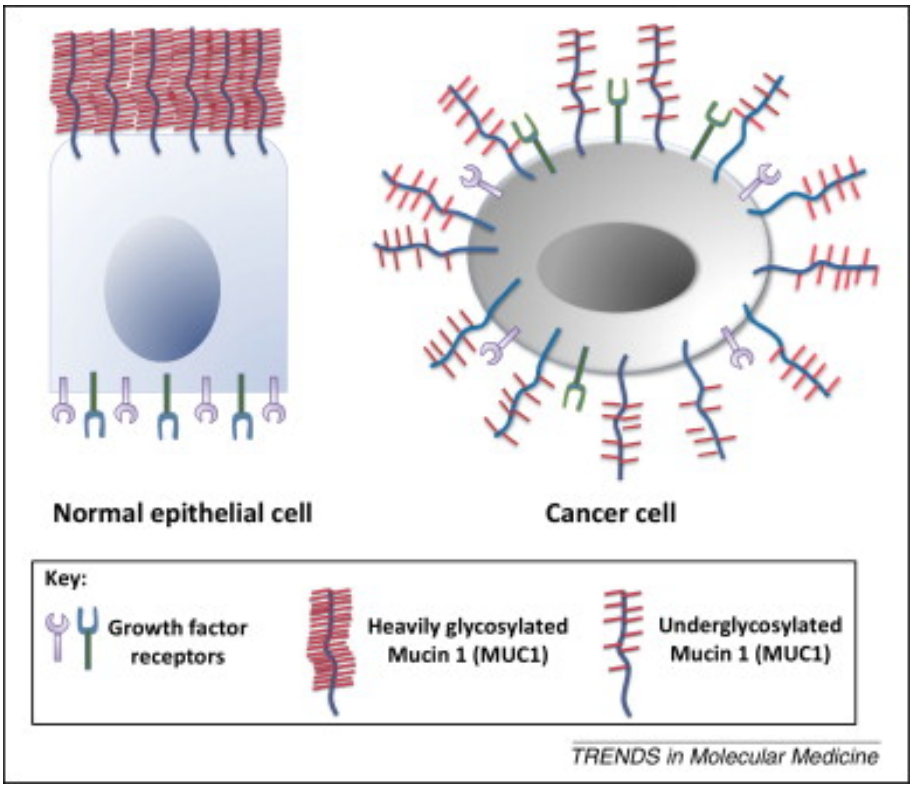

Figure 3: Changes in Muc1 During Cancer Progression. Tumor associated Muc1 is hypoglycosylated and overexpressed over the whole cell surface. Reproduced from Nath et al (2014) with permission.

Muc1 expression correlates with aggressive, metastatic cancers and there are numerous mechanisms with Muc1 involvement in metastatic progression. It has been hypothesized that the upregulation of Muc1 affects cancer cell invasion, proliferation, and survival by reducing cell-cell adhesion and cell-extracellular matrix adhesion [14]. To give a couple of examples, first, Muc1-

mediated cancer cell survival effects are believed to be through the interaction of Muc1-C with various signaling molecules, which lead to alterations in gene transcription that promote survival [15]. Second, Muc1 has been shown to promote growth of these cancer cells through its interaction with various growth factor receptors, leading to uncontrolled proliferation [16]. Lastly, Muc1 suppresses apoptosis of cancer cells through its numerous interactions with pathways involved in cell death, including p53, FOXA3a, and NF-kB [16]. These examples only illustrate three mechanisms, but there are numerous roles Muc1 plays in the pathogenesis of cancer, both already investigated and have yet to be identified.

Anti-Muc1 Antibody

Several anti-Muc1 antibodies have been developed over the past 40 years. The differences in O-glycosylation patterns in the VNTR regions between tumor associated Muc1 and normal epithelial cell associated Muc1 lead to the presentation of different epitopes on Muc1[17]. In cancer cells, the hypoglycosylation and truncated sugar chains of Muc1 expose the formerly masked peptide backbone to antibodies for attack [18,19] (see Figure 4). Because of the differences in Muc1 properties, the anti-Muc1 antibody is able to discriminate and specifically attack Muc1 positive cancer cells. The antibodies on their own have not proven to be an effective form of cancer treatment for many reasons. In an international workshop on monoclonal antibodies against Muc1 in 1996, results showed that antibodies recognizing the same peptide region had different binding patterns [20,21]. Further studies have shown that the antibodies are not sufficiently discriminating between normal and tumor associated Muc1 [21]. We do recognize this problem and have addressed it by generating new antibody sequences at the complementarity-determining regions that have been shown in the literature to have enhanced specificity for tumor associated Muc1.

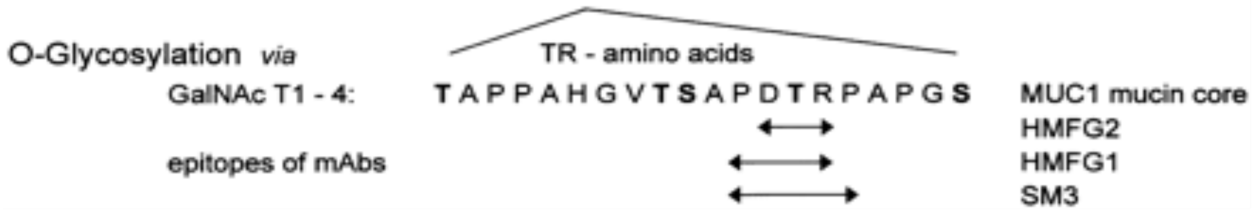

Figure 4: Antibody Binding to Muc1 Sequence. When Muc1 becomes hypoglycosylated and its sugar chains become truncated, the VNTR region becomes exposed for anti-Muc1 antibody binding, shown with HMFG2, HMFG1, and SM3. Reproduced from Franke et al (2001) with permission.

Antibody-Drug Conjugates (ADCs)

In this project, we aim to develop a therapeutic molecule that combines the specificity approach of antibodies with the toxicity approach of chemotherapy in order to develop an antibody-drug conjugate. There are three components of an antibody-drug conjugate. First, an antibody that has specificity for an antigen substantially expressed on tumor cells, with limited expression on normal tissues. Second, a linker that is stable enough so that the drug is not prematurely released systemically, but not so much that the drug can still be cleaved off once intracellularly to induce its cytotoxic effects. Lastly, a drug that is often too toxic to be used on its own that has high potency at low concentrations and high cytotoxicity [22,23] . The goal in generating an antibody-drug conjugate is to take two therapies that have not proven to be efficacious and safe on their own, couple them together and create a new target therapy. ADCs account for the advantages and disadvantages of each treatment on their own by combining the precision capabilities of monoclonal antibodies in targeting the cell surface of cancer cells and the nonspecific toxicity characteristics of chemotherapy drugs [24] (see Figure 5).

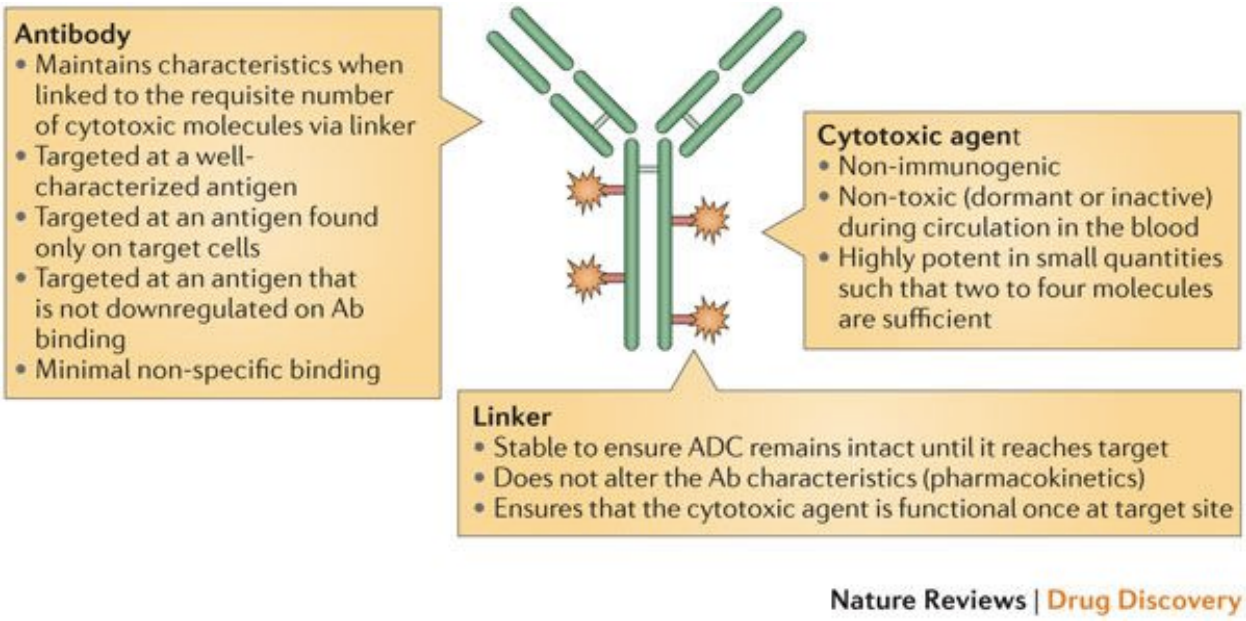

Figure 5: Antibody-Drug Conjugate Components. ADCs link the targeting aspect of monoclonal antibodies with the cytotoxic capabilities of chemotherapy drugs. Reproduced from Million et al (2013) with permission.

Figure 6 illustrates the mechanism of action of antibody-drug conjugates. First, the ADC is delivered systemically and circulates through the bloodstream. Again, the linker must be stable in this environment so that the cytotoxic drug is not prematurely released. Next, the antibody reaches its target site and binds to the antigen on the surface of the cancer cell. This causes the ADC to become internalized via a clathrin-coated pit mechanism into the endosome-lysosome pathway. Once internalized, the new internal conditions destabilize the linker, resulting in cytotoxic drug release into the cytoplasm. Lastly, the free drug in its active form is able to bind to its target site in order to induce apoptosis of the tumor cell [25,26].

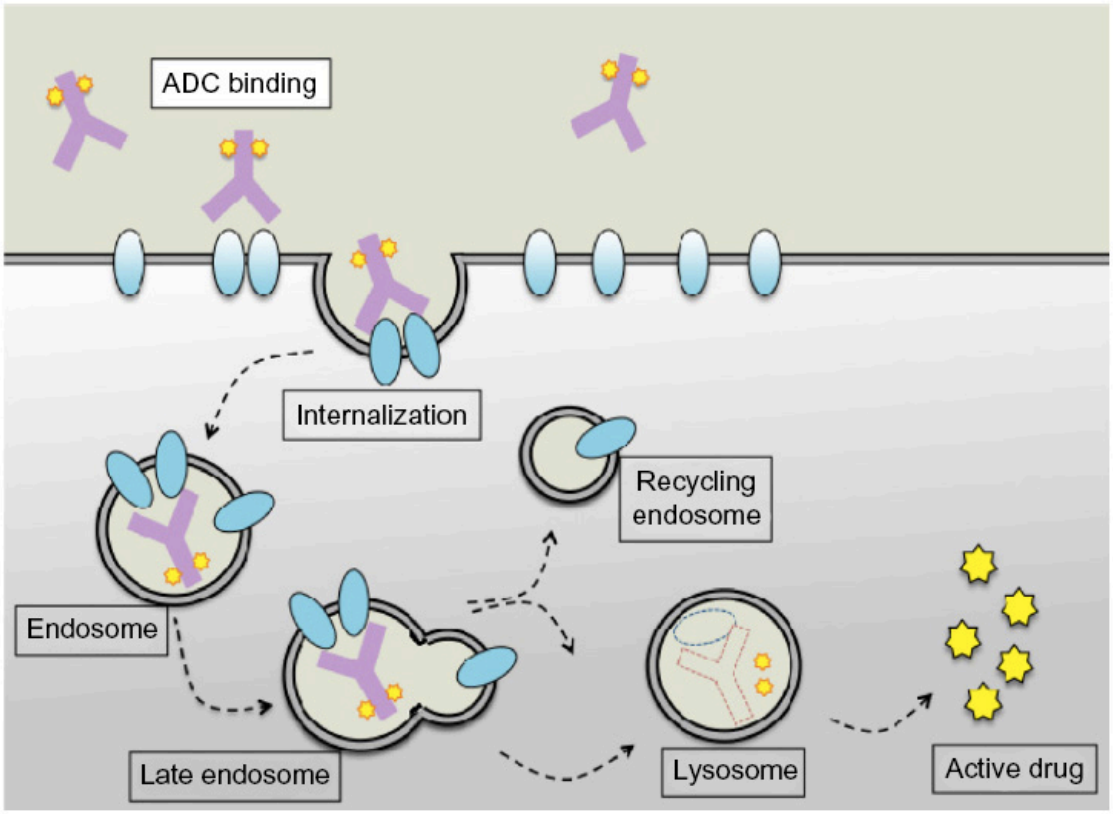

Figure 6: ADC Mechanism of Action. The mechanism of the ADC proceeds as follows: antibody binding target antigen on cancer cell, internalization through the endosome-lysosome pathway, cytotoxic drug release, and then cell death. Reproduced from Scotti et al (2015) with permission.

Solution: Anti-Muc1-DM1

In this project, we aim to develop an antibody-drug conjugate that targets Muc1 on cancer cells by coupling an anti-Muc1 antibody with mertansine (DM1). Mertansine is a maytansinoid that kills cells via blocking cell division by interacting with tubulin to disrupt microtubule formation and depolymerize already formed microtubules [27,28]. On its own, mertansine has not been proven to be effective as a cancer treatment because of its high systemic toxicity side effects, resulting in a low therapeutic index [28,29]. DM1 has been an extensively used candidate for ADCs, including in a current HER2-targeting ADC (Herceptin), given its cytotoxicity, potency, solubility profile, and

internalization qualities [28] . We hope to expand mertansine's therapeutic window by using it as the cytotoxic payload of our antibody-drug conjugate.

Recent studies have shown that noncleavable linkers are a better choice over cleavable linkers because of their stability at physiological pH and their stability in the circulation [28,30] . The noncleavable linker does not have an obvious drug release mechanism; some of the noncleavable linkers rely on differences in glutathione concentrations intracellularly and extracellularly in order reduce disulfide bonds for drug release [31]. SMCC (succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate) is the noncleavable linker used as the crosslinking agent in this project. The linker has often been used in conjugations with DM1 due to its increased stability and half-life in plasma. Linking the drug to the antibody involves conjugation to the exposed amino groups on the lysine residues of the anti-Muc1 antibody (see Figure 6).

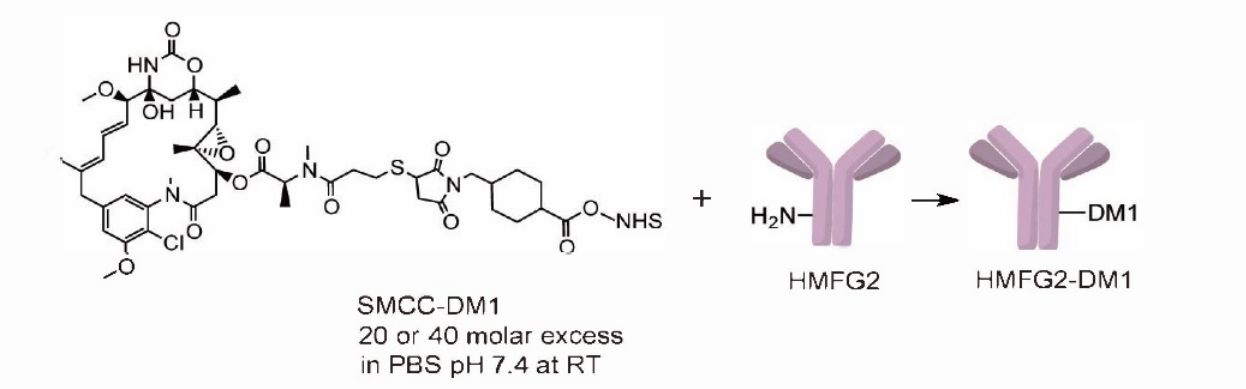

Figure 7: ADC Conjugation with SMCC and DM1. Anti-Muc1 antibody is conjugated to the microtubule inhibitor, DM1, with the SMCC linker, a noncleavable disulfide bond linker. Reproduced from Lee (2017) with permission.

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Our aim is to build upon advancements made in the last thirty years with several anti-Muc1 antibodies being developed that display high affinity, specificity, and stability *in vitro*. However, these antibodies have not proved to be successful as cancer therapeutics because they have not shown significant decreases in tumor growth or survival *in vivo*. We hypothesize that an antibody-drug conjugate developed to target tumor-associated Muc1 and deliver a cytotoxic drug will destroy target cancer cells. By using an ADC, we intend to enhance the anti-cancer effects of the antibody by conjugating a cytotoxic drug to it. Further, we intend to reduce the toxicity of the chemotherapy drug by antibody-mediated targeted delivery. Our hypothesis for this project is that the combination of the anti-Muc1 antibody and cytotoxic agent produces an improved efficacy over antibody alone and reduced toxic side effects of drug alone, ultimately leading to the destruction of tumor-associated Muc1 cancer cells.

Specific Aims

I. Design and synthesize an anti-Muc1 antibody-drug conjugate
   a. Design constructs for an antibody that specifically targets Muc1 on both its N-terminal region and its C-terminal region
   b. Synthesize an anti-Muc1 ADC using a SMCC-DM1 conjugation approach and optimize its reaction

II. Investigate the in vitro anti-cancer effects of the anti-Muc1 antibody-drug conjugate compared to its parent antibody

a. Use flow cytometry to study the binding of the anti-Muc1 antibody construct to cancer cells versus noncancer cells
b. Measure metabolic activity of cancer cells treated with the anti-Muc1 ADC and its parent antibody to determine the drug's cytotoxicity profile
c. Further characterize the anti-Muc1 ADC to determine the drug-to-antibody ratio using mass spectrometry

MATERIALS AND METHODS

Antibody Synthesis

Three anti-Muc1 antibodies (HMFG2, 12E, and SM3) were generated using the backbone of Trastuzumab (Herceptin). The plasmid DNA-encoding pVITRO1-Trastuzumab-IgG1/k was obtained from Addgene (plasmid #61883 deposited by Andrew Beavil). Changes were made in the V_H and V_L regions of Trastuzumab in order to generate the new anti-Muc1 antibodies. HMFG2 was generated using V_H accession number CAN99757.1 and V_L accession number AAB97462.1 [32]. 12E was generated using V_H accession number ACH99176.1 and V_L accession number AAG28706.2 [33]. SM3 was generated using V_H accession number AAB97461.1 and V_L accession number AAB97462.1 [32].

DNA Isolation

The anti-Muc1 antibody gene is transformed into Invitrogen One Shot TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). 50 uL of the cells is combined with 1-5 uL of DNA and the mixture is allowed to incubate on ice for 30 minutes. The cell/DNA mixture is heat-shocked at 42°C for 30 seconds and then placed on ice. 250 uL of SOC media is added to each tube and grown for one hour while shaking at 225 rpm at 37°C. The cells are then plated on an LB agar plate containing hygromycin B gold (InvivoGen, San Diego, CA) at a concentration of 100 ug/mL and incubated overnight at 37°C.

The following day, one colony is selected from the LB agar plate and grown in 5 mL of LB media containing 100 ug/mL of hygromycin B gold for three hours while shaking at 225 rpm at 37°C. The mixture is then transferred to a 1.2 L of LB media containing 100 ug/mL of hygromycin B

gold in a 2 L flask and allowed to grow overnight. The bacterial cells are harvested through centrifugation at 4700 x g for 10 minutes at 4°C. The DNA is isolated using a NucleoBond Xtra EF plasmid purification kit (Machery-Nagel, Bethlehem, PA). DNA concentration is measured using the Synergy 4 Multi-Detection Microplate Reader (BioTek, Winooski, VT).

Transfection

Expi293 cells (ThermoFisher, Waltham, MA) are subcultured to a concentration of about 5 x 10^6 cells/mL in a 1L flask. The Expi293 cells are spun down at 200 rpm for 6 minutes and the media is aspirated. The cells are diluted to a concentration of 600 x 10^6 cells in 200 mL fresh media in a 1L flask. 200 uL of DNA at a concentration of 1 mg/mL is combined with 12 mL Opti-MEM (ThermoFisher, Waltham, MA) and incubated for 5 minutes. 640 uL of ExpiFectamine 293 Reagent (ThermoFisher, Waltham, MA) is combined with 11.2 mL Opti-MEM and incubated for 5 minutes. The ExpiFectamine 293 Reagent is a cationic lipid transfection reagent that binds the negatively charged DNA backbone. The complex enters the cell through endocytosis and then diffuses through the cytoplasm to enter the nucleus for gene expression. The two mixtures are combined and incubated for 20 minutes to form the transfection complex. The ExpiFectamine 293/plasmid DNA complex is slowly added to the 1L flask of Expi293 cells. The cells are shaken at 125 rpm and incubated in a 37°C and humidified (8% CO_2) incubator. At 18-22 hours post transfection, 1.2 mL of Expifectamine 293 Enhancer 1 (ThermoFisher, Waltham, MA) and 12 mL of Expifectamine 293 Enhancer 2 (ThermoFisher, Waltham, MA) is added to the flask and then returned to the incubator. We conducted a transfection optimization protocol and found that the cells are to be taken out on day 7 in order to produce the highest protein yield. On day 7, the cells are centrifuged at 4700 x g for 30 minutes and the supernatant is filtered through a 0.2 um filter.

Purification

The proteins are purified using fast protein liquid chromatography (FPLC). Affinity chromatography using protein A, a cell wall protein that has high specificity for the Fc region of the immunoglobulin, is used for purification. Protein concentration is quantified using Pierce BCA Protein Assay Kit (ThermoFisher, Waltham, MA) and measured using the BioTek Synergy 4 Multi-Detection Microplate Reader.

Antibody-Drug Conjugate Synthesis

Anti-Muc1 antibody is combined with DM1-SMCC (Cayman Chemical Company, Ann Arbor, MI) at a drug:Ab molar ratio of 10:1, 20:1, and 40:1 (referred later on as R10, R20, and R40). First, the antibody is diluted with PBS at a ratio of 3 mL PBS: 1mL antibody. While the mixture is stirring, DMSO (Sigma-Aldrich, St. Louis, MO) is added so that the percent DMSO is equal across all three forms of ADC. The DM1-SMCC stock solution is added quickly at a volume corresponding to its molar ratio. The mixture is stirred for 2-4 hours at room temperature away from light.

After the conjugation reaction, the ADC is dialyzed against PBS using a 20,000 molecular weight cut-off dialyzer cassette (ThermoFisher, Waltham, MA). The mixture is left to dialyze for 24-48 hours at room temperature away from light. The product is sterilized by filtration using a 0.2 um filter. The final ADC is then purified using FPLC with the same procedure as described above.

Cell Culture

The following cells (normal, lung cancer, and breast cancer) were purchased from American Type Culture Collection (ATCC, Manassas, VA): CCD-19Lu, Calu 3, H1299, H1975, A549, HCC827, MCF7, MDA-MB 231, and SkBr3. Upon thawing the cells, the suspension is centrifuged at 200 rpm for 6 minutes in order to remove the DMSO. The cells are then placed in their respective media that contains 1% of 100 U penicillin / 0.1 mg/mL streptomycin solution (ThermoFisher, Waltham, MA) and 10% of fetal bovine serum (FBS) (ThermoFisher, Waltham, MA). The cells are plated on a 175 cm^2 flask with 25mL of its complete media. Cells are incubated at 37°C and placed in a humidified (5% CO2) incubator. The cells are allowed to grow until they reach about 80% confluency. At this time, the cells are subcultured.

Antibody Binding Assay

To determine how well the Anti-Muc1 antibody binds to cancer cells, a fluorescence imaging assay is conducted using fluorescein isothiocyanate (FITC) (ThermoFisher, Waltham, MA). FITC is a fluorescein molecule with a molar mass of 473.4 g/mol that is reactive with the amines present on the surface of the Anti-Muc1 antibody. 1.5-3.0 mg of the Anti-Muc1 antibody is added to a vial with a stir bar. While the mixture is spinning, the fluorescein dye (15-30 uL) is added at a ratio of 30 moles dye to 1 mole of antibody. The mixture incubates for 2 hours while stirring and away from light. After binding is allowed to occur, the mixture is dialyzed against PBS using a 20,000 molecular weight cut-off dialyzer cassette. The mixture is left to dialyze overnight at room temperature away from light.

Flow cytometry cell sorting (FACS) is used to detect the FITC signal, which has excitation and emission spectrum peak wavelengths at 495nm and 519nm. Samples are prepared for analysis just prior to FACS measurement. Samples are made by combining 1 x 10^5 cells (CCD19Lu, Calu 3, H1299, H1975, A549, HCC827, MCF7, MDA MB 231, and SkBR3) and 10 ug of DNA. The samples incubate for one hour at room temperature and the binding characteristics are quantified using the LSR II flow cytometer (BD Biosciences, San Jose, CA).

Cytotoxicity Assay

On the first day of the preparation for the cytotoxicity experiment, the cells (CCD19Lu, Calu 3, H1299, H1975, A549, HCC827, MCF7, MDA MB 231, and SkBR3) are plated in a 96 well plate. Each well contains 5 x 10^3 cells in 150 uL of their respective media. The cells are allowed to incubate overnight at 37°C. The next day, the Anti-Muc1 antibody-drug conjugate is introduced. The ADC is added to each cell line at a final concentration ranging from 0.0005 ug/mL to 50 ug/mL.

At 24 hours post ADC treatment, cell death is quantified using the MTT assay kit (Abcam, Cambridge, UK). MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) is a dye that turns purple when NADPH-dependent cellular oxidoreductase enzymes reduce it. Therefore, those wells that have more living cells, thus more metabolic activity, will exhibit a darker purple coloring. First, all media is aspirated from the wells carefully and without scratching the bottom surface. The MTT solution is prepared with complete media so that the MTT is at a concentration of 0.5 mg/mL. 150 uL of the MTT solution is added to each well and the plates are allowed to incubate for 3-4 hours at 37°C. Following incubation, each well is aspirated. 150 uL of DMSO is

added to each well and mixed well. The absorbance is recorded at an OD of 590nm using the BioTek Synergy 4 Multi-Detection Microplate Reader. IC_{50} and statistical analysis is conducted using GraphPad Prism 8 software (GraphPad, San Diego, CA).

Mass Spectrometry Analysis

A sample of Anti-Muc1 antibody DNA at 1 mg/mL was sent to Analytical and Biological Mass Spectrometry at the University of Arizona and analyzed using electrospray ionization mass spectrometry (ESI/MS).

RESULTS

Transfection Optimization and Gel Verification

At the beginning of antibody development, we produced the three Anti-Muc1 antibodies (HMFG2, SM3, and 12E) using both ExpiCHO (Chinese hamster ovary cells) and Expi293 (human embryonic kidney cells) cells. In order to optimize our transfection protocol, we compared the protein yield from using each cell line expression system. Following purification using FPLC, the protein yield was determined. Table 2 shows that the Expi293 expression system was found to be superior to ExpiCHO in terms of protein yields, so we decided to use that system for the rest of the project.

The native and SDS gels in Figures 7 and 8, respectively, also compare the two expression systems in terms of protein purity. The ExpiCHO cell lines produced proteins that were not as pure with more breakdown products. Further verifying our transfection and purification procedures, we see

the antibodies coming out at their respective molar masses (approximately 148,000 Da) in the native gel and the heavy and light chains coming out in the denatured gel. These gels provided more evidence to proceed with using the Expi293 expression system for the rest of the project.

Antibody	ExpiCHO Yield (mg/L)	Expi293 Yield (mg/L)
12E	7.22	6.00
HMFG2	7.72	10.3
SM3	0.93	3.35

Table 2: Anti-Muc1 Yield Using Two Expression Systems. Three anti-Muc1 antibodies were produced using two different expression systems for transfection, ExpiCHO and Expi293, and their yields were compared.

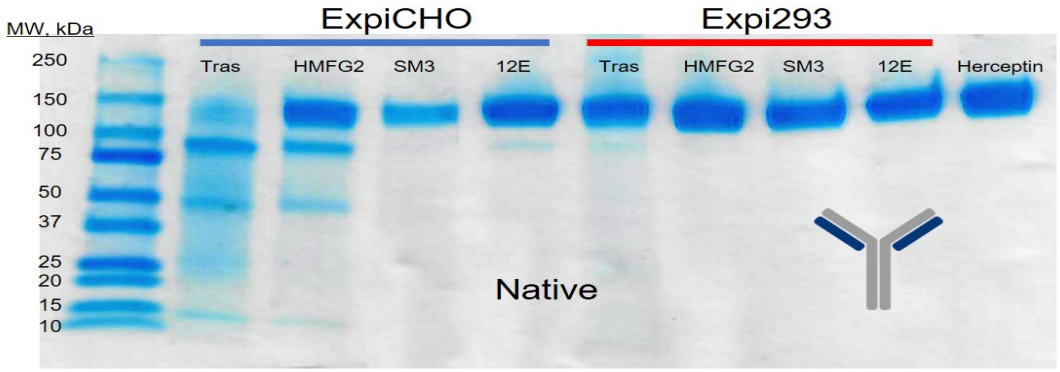

Figure 8: Native anti-Muc1 Gel. Anti-Muc1 is coming out at its 148kDa molar mass with increased purity using the Expi293 expression system.

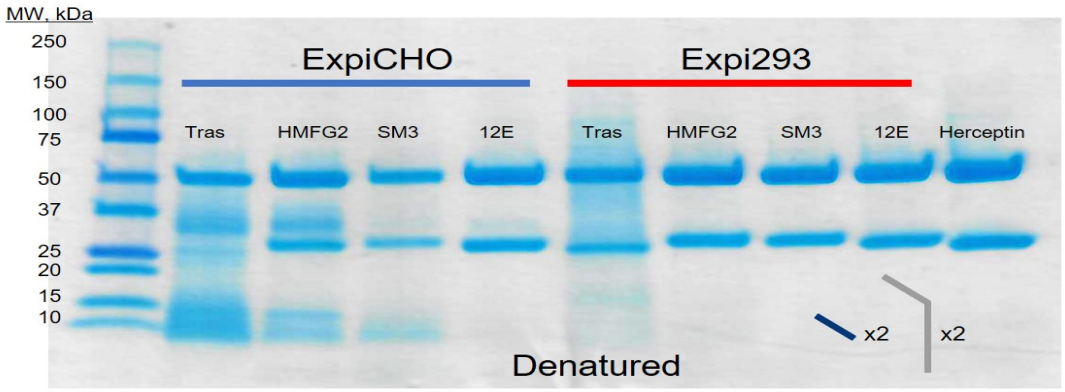

Figure 9: SDS anti-Muc1 Gel. With the disulfide bonds reduced between the heavy and light chains, the respective bands are visualized.

Antibody Binding Assay

To test how well the anti-Muc1 antibody binds to the cancer cells compared to normal cells, we used a FITC conjugation approach and measured the fluorescence using flow cytometry. A greater shift to the right in the FITC histogram illustrates a greater percentage of anti-Muc1 expression on the cell. In comparing our three synthesized anti-Muc1 antibodies, we noticed that there was a greater percentage of HMFG2 expressed on the cancer cells, compared to 12E and SM3. For example, in looking at MCF7 cells (breast adenocarcinoma), we saw that 75.1% of HMFG2 binding to Muc1 expressed on the cell membrane, whereas for the SM3 and 12E antibodies, there was only 3.47% and 32.9% of binding to Muc1. This trend was seen throughout most of the other cancer cell lines. Given these binding characteristics, we concluded that the HMFG2 antibody would be the most efficacious compared to 12E and SM3, so we decided to further investigate only the HMFG2 antibody for DM1-SMCC conjugation experiments.

The normal lung cells, CCD-19Lu, exhibited the least amount of anti-Muc1 expression throughout its surface, which is crucial to the theories behind antibody-drug conjugates. We hoped to develop a drug that specifically targets and kills cancer cells, while showing little toxicity for normal, non-cancerous cells.

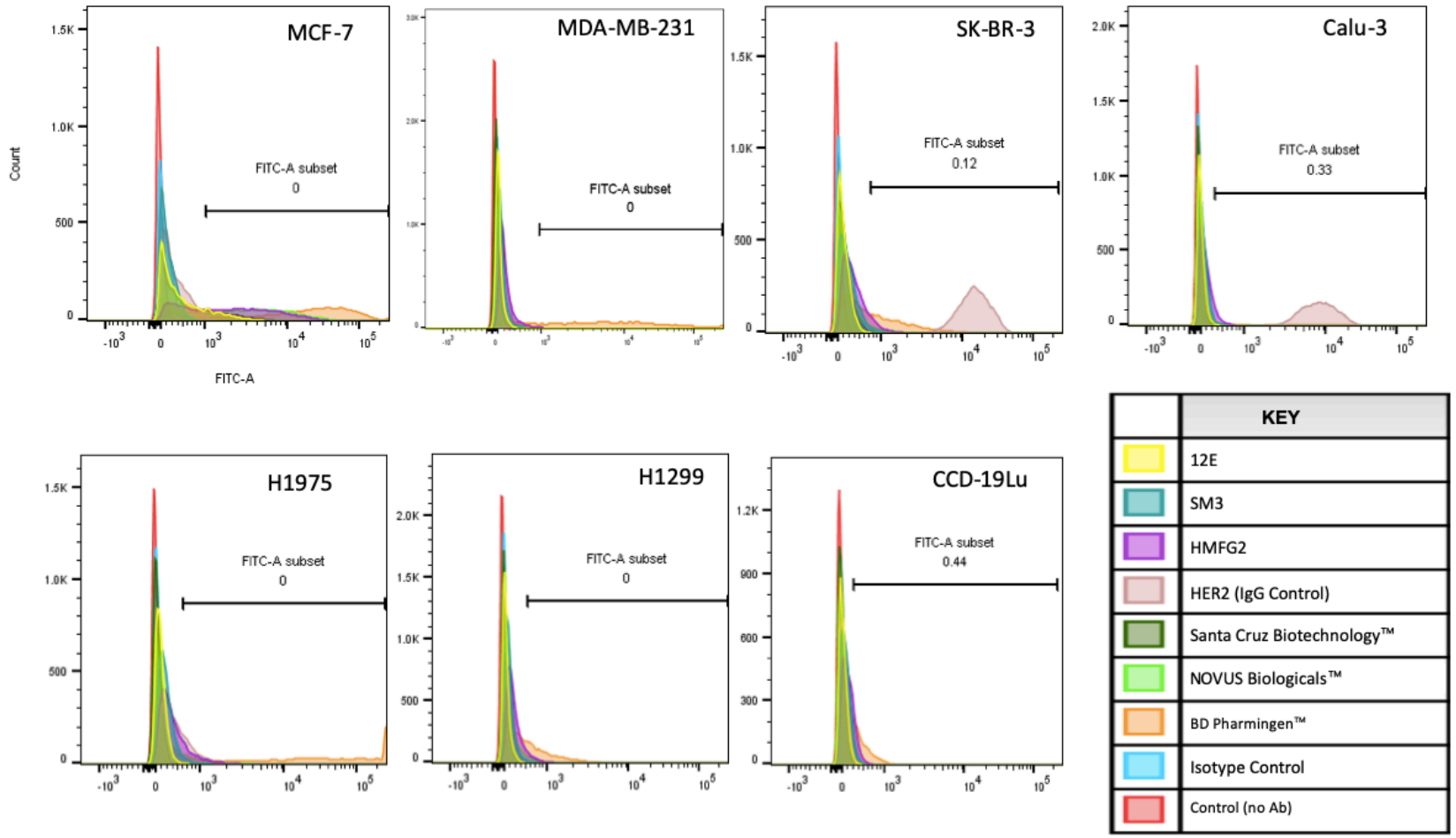

Figure 10: FITC/FACS Antibody Binding Assay. A mixture of FITC bound to anti-Muc1 antibody was allowed to incubate with cancerous and noncancerous cells for one hour. Fluorescence was measured and compared to that measured from the isotype control.

Cell Line	Origin	Type	Commercialized MUC1			Synthesized MUC1			IgG Control (HER2)
			BD Pharmingen™	NOVUS Biologicals™	Santa Cruz Biotechnology ™	HMFG2	SM3	12E	
CCD-19Lu	Lung	Normal	16.4	0.55	0.18	2.17	1.13	0.70	0.16
Calu 3	Lung	Adenocarcinoma	20.2	6.40	1.77	15.6	5.33	2.69	99.6
H1299	Lung	NSCLC	71.5	23.5	2.36	39.5	13.3	4.39	12.7
H1975	Lung	NSCLC	68.2	27.5	1.24	46.2	23.5	5.60	53.0
A549	Lung	Adenocarcinoma	1.51	1.47	0.53	8.43	4.84	2.58	5.35
HCC827	Lung	Adenocarcinoma	27.3	7.47	0.75	12.5	5.39	2.87	3.68
MCF7	Breast	Adenocarcinoma	83.8	75.5	37.5	75.1	3.47	32.9	18.2
MDA-MB-231	Breast	Adenocarcinoma	83.2	7.18	0.30	7.99	1.74	0.83	0.42
SkBR3	Breast	Adenocarcinoma	45.2	10.1	1.58	15.5	5.93	1.71	99.8

Table 3: Muc1 Expression Summary. Percentage of Muc1 expression on each cell type was reported from FITC/FACS analysis. An ideal anti-Muc1 antibody binds to cancerous cell lines expressing Muc1, but not to the noncancerous CCD019Lu cells

Anti-Muc1-DM1 Cytotoxicity

The cytotoxicity of anti-Muc1 antibody conjugated to the SMCC linker and mertansine chemotherapy drug was investigated using an MTT colorimetric assay that assesses cell activity through the presence of NAD(P)H-dependent cellular oxidoreductase enzymes. The more of these enzymes present, the more MTT is reduced to its insoluble form, which is purple in color. The greater the absorbance at 590 nm, the greater number of viable cells, so the less cytotoxic the ADC.

The cytotoxicity assay was run for a total of three trials. Among all of the trials, we noticed that the ADC did not induce significant cytotoxicity on cancer cells until 48-72 hours of incubation. In all three trials, we saw that the R20 ADC had a considerable decrease in IC_{50} compared to the antibody alone. Furthermore, we saw that the R40 ADC had a decrease in IC_{50} compared to the R20 ADC.

Looking at the CCD-19Lu, normal lung cells, we saw that the ADC does not exhibit significant cytotoxic effects until it reaches a large concentration. In all conditions, the IC_{50} was considerably larger for the treatments against the CCD-19Lu cells compared to the cancer cells. For these cells, we also saw that DM1 has an effect at lower concentrations. It is important to remember that the mechanism of DM1, or mertansine, arrests the cell cycle during division via microtubule inhibition. The drug is killing off the normal, noncancerous cells because they also contain microtubules. Further, the drug has a greater effect on cells that have faster proliferative rates, meaning it is less efficacious in normal cells. This is why we hoped to develop an ADC that combines this toxicity effect with the specificity effect of the anti-MUC1 antibody. The cancer cells that the ADC seemed to have the greatest cytotoxic effect were: H1975, HCC827, and MCF7.

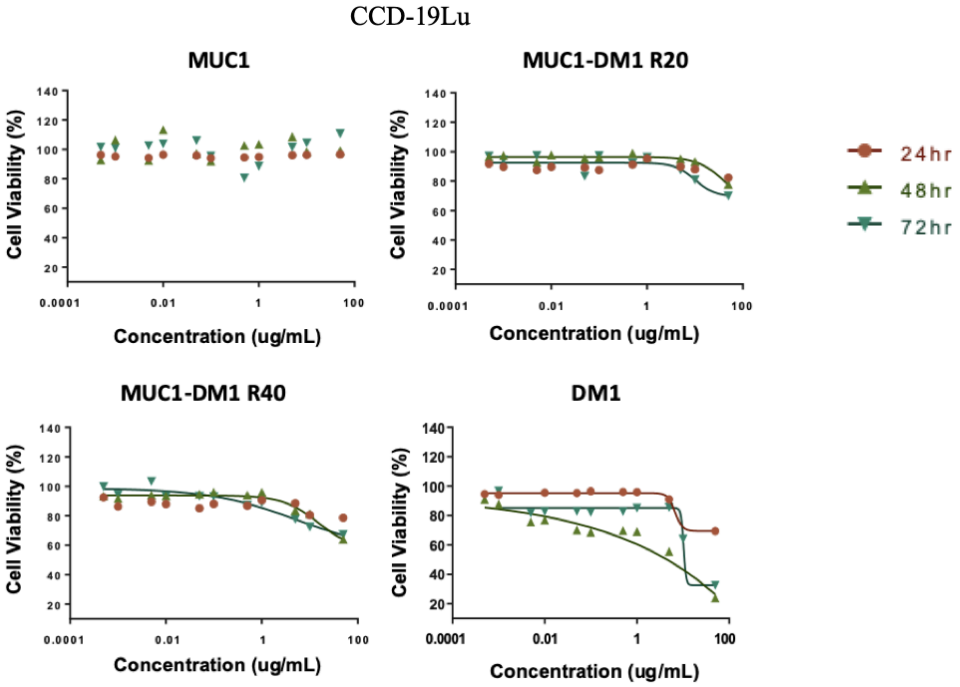

Figure 11: HMFG2 ADC Cytotoxicity Assay on CCD-19Lu Cells. Cytotoxicity effect of ADC on normal lung cells.

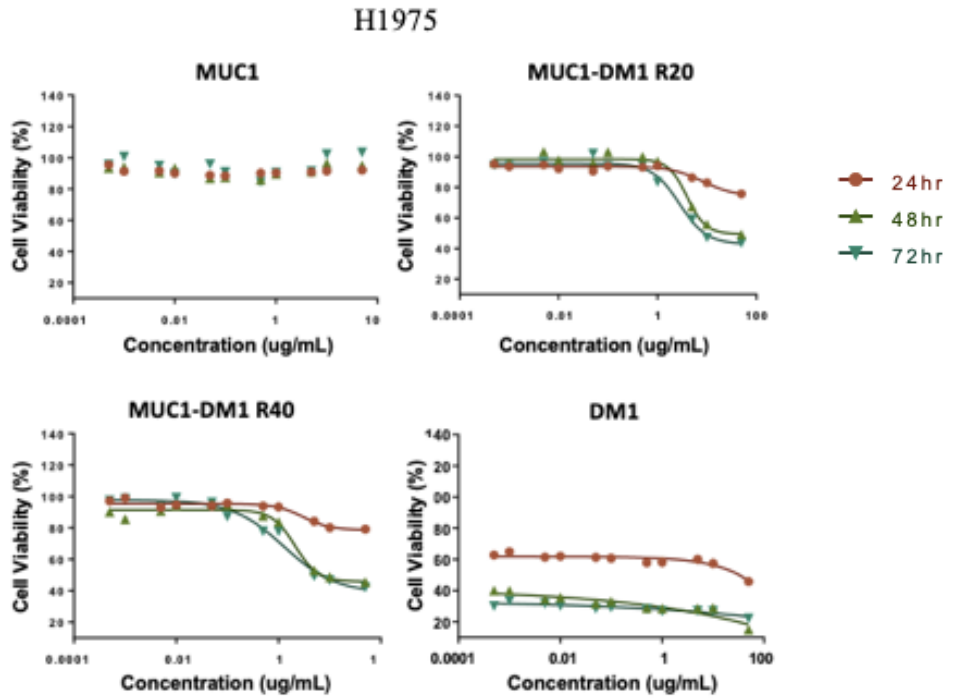

Figure 12: HMFG2 ADC Cytotoxicity Assay on H1975 Cells. Cytotoxicity effect of ADC on non-small cell lung cancer cells.

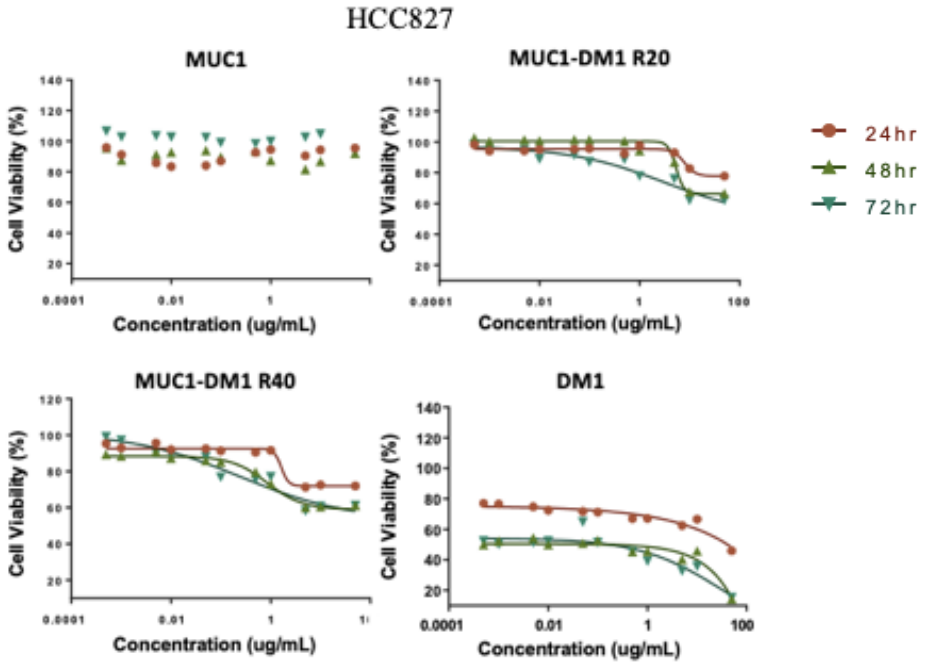

Figure 13: HMFG2 ADC Cytotoxicity Assay on HCC827 Cells. Cytotoxicity effect of ADC on lung adenocarcinoma cells.

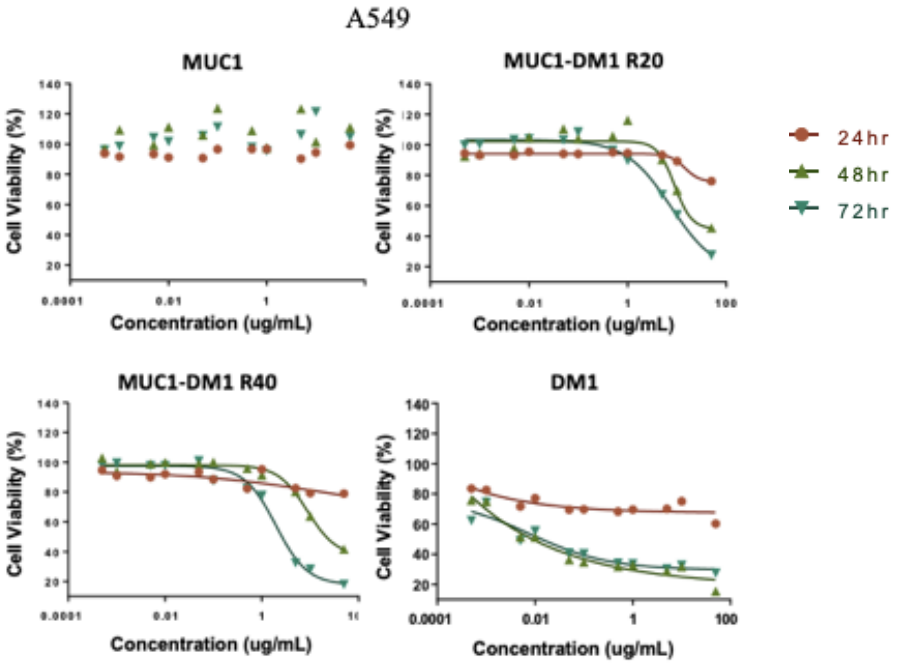

Figure 14: HMFG2 ADC Cytotoxicity Assay on A549 Cells. Cytotoxicity effect of ADC on lung adenocarcinoma cells.

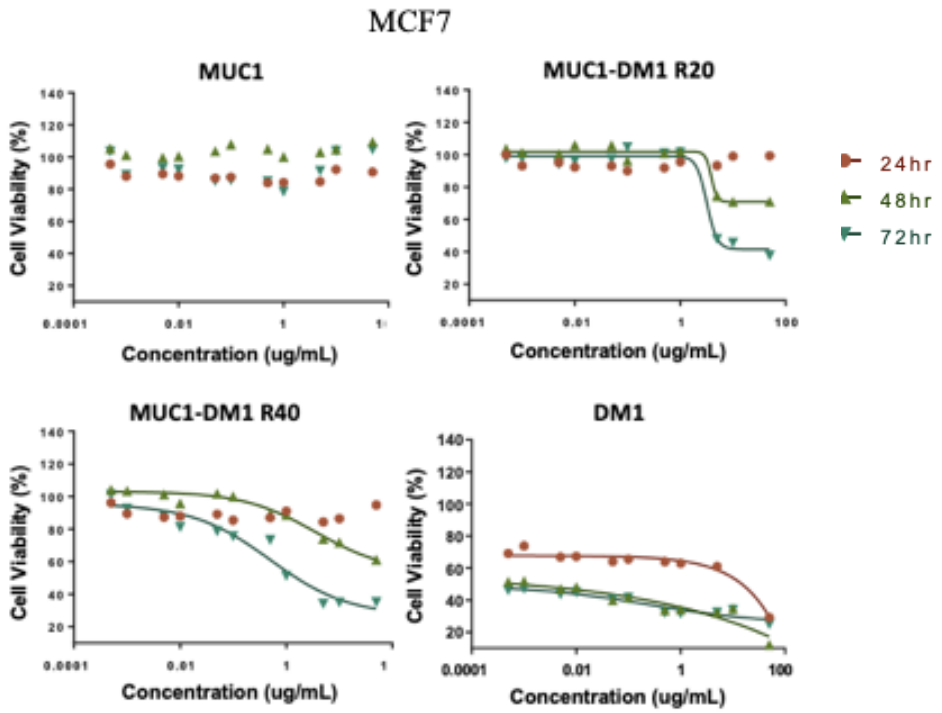

Figure 15: HMFG2 ADC Cytotoxicity Assay on MCF7 Cells. Cytotoxicity effect of ADC on breast adenocarcinoma cells.

In vitro HMFG2-DM1 (MUC1-DM1) IC50 (ug/mL)					
Cell Line	**Time (hr)**	**DM1**	**HMFG2**	**HMFG2-DM1 R20**	**HMFG2-DM1 R40**
CCD-19Lu (Normal)	24	-	>10	>10	>10
	48	-	>10	>10	>10
	72	-	>10	>10	>10
H1975 (Lung)	24	-	>10	6.93 – 8.13	2.13 – 4.06
	48	-	>10	3.95 – 4.21	2.10 – 3.42
	72	-	>10	2.79 – 3.74	1.17 – 3.14
HCC827 (Lung)	24	-	>10	6.93 – >10	1.63 – >10
	48	-	>10	5.37 – 5.69	0.85 – 3.09
	72	-	>10	3.15 – 5.02	0.24 – 2.80
A549 (Lung)	24	-	>10	>10	>10
	48	-	>10	>10	>10
	72	-	>10	3.29 – 9.39	1.96 – 9.01
MCF-7 (Breast)	24	-	>10	5.99 – >10	3.42 – >10
	48	-	>10	3.89 – 4.39	3.26 – 3.60
	72	-	>10	3.04 – 4.17	0.50 – 3.18

Table 4: *In vitro* HMFG2-DM1 IC_{50}. Statistical analysis from the MTT cytotoxicity assays presented in Figures 10-14 was conducted using Graphpad Prism 6. The IC_{50} values are given in ranges because the assay was repeated three times.

Mass Spectrometry

Using electrospray ionization mass spectrometry, the number of mertansine molecules conjugated on HMFG2, the drug-to-antibody ratio (DAR), was quantified. For the unconjugated HMFG2 antibody, the DAR is 0. For the ADCs, the DARs for HMFG2-DM1 R10, HMFG2-DM1 R20, and HMFG2-DM1 R40 are 1.25, 1.79, and 2.34, respectively.

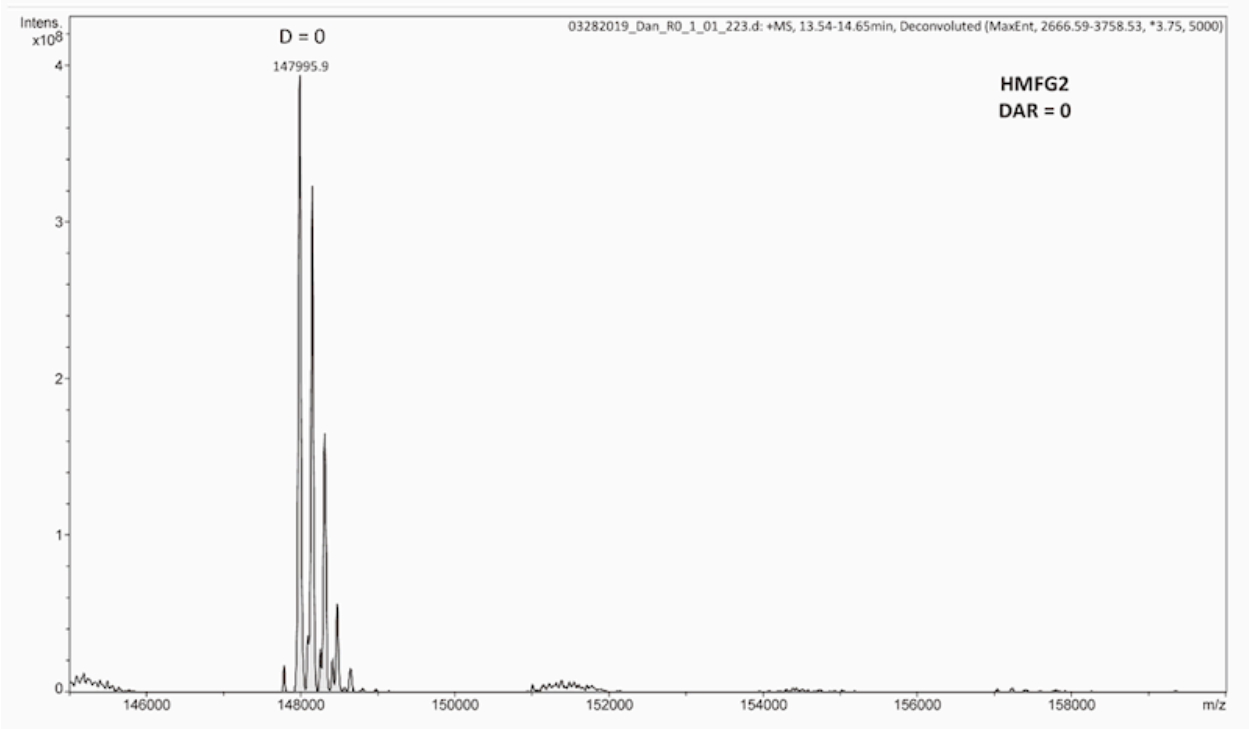

Figure 16: Mass Spectrometry of HMFG2.

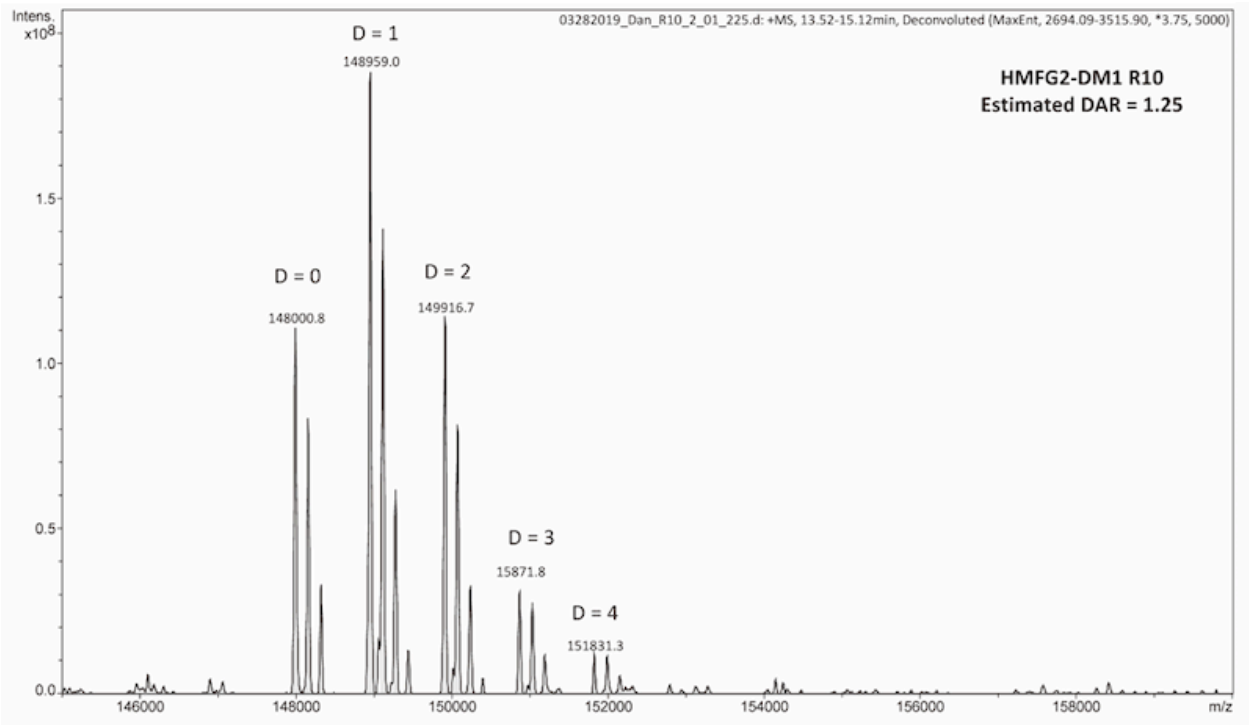

Figure 17: Mass Spectrometry of HMFG2-DM1 R10.

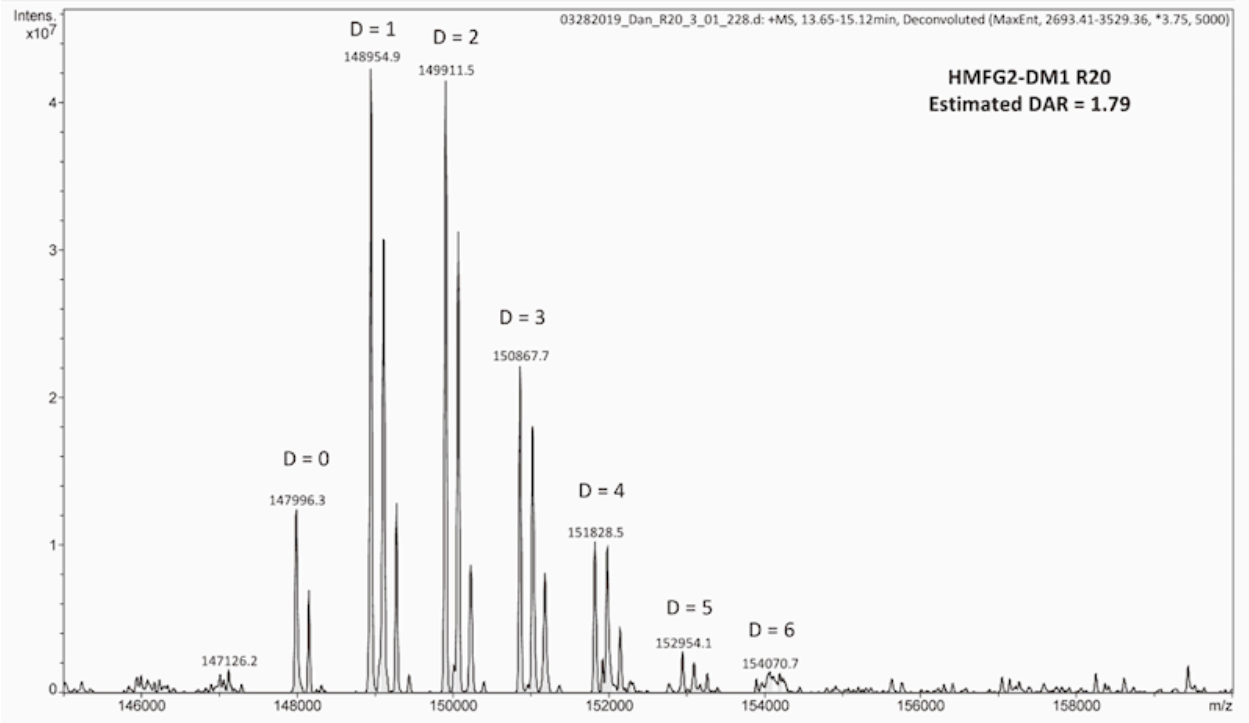

Figure 18: Mass Spectrometry of HMFG2-DM1 R20.

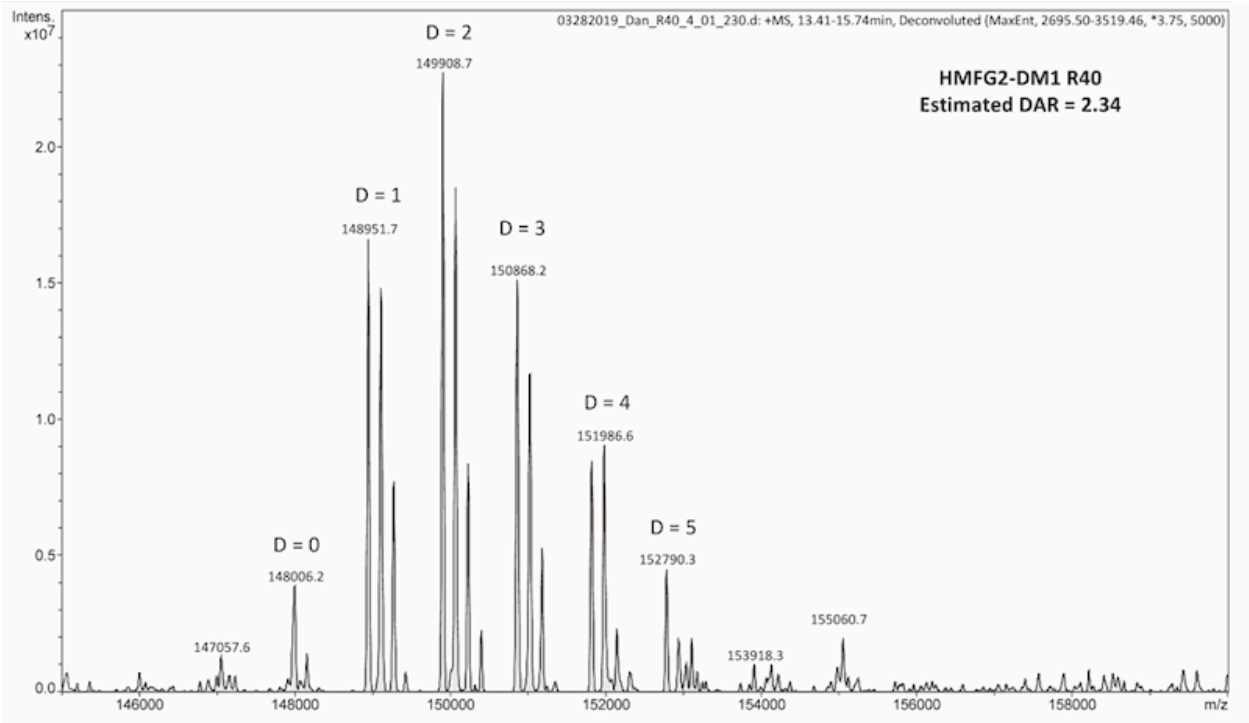

Figure 19: Mass Spectrometry of HMFG2-DM1 R40.

DISCUSSION

Antibody Binding Assay

In conducting the antibody binding assay, our goals were to investigate the binding properties of the synthesized antibodies to both cancerous cells and noncancerous cells and compare those results to three commercialized anti-Muc1 antibodies that are currently on the market. To be an ideal candidate for the antibody-drug conjugate, we wanted our anti-Muc1 antibody to be specific to cancer cells. This was seen with increased binding percentage towards Muc1 on the cancerous cells and a decreased binding percentage towards Muc1 on the noncancerous CCD19-Lu cells. With the BD Pharmingen™ commercialized antibody, we see in Table 3 that the antibody was binding to the Muc1 on the H1299, H1975, MCF7, and MDA-MB-231 cancerous cell lines at a significant proportion. However, this antibody is not specific to cancer cells because 16.4% of CCD19-Lu normal cells are expressing Muc1 that is recognized by the BD Pharmingen™ antibody. The BD Pharmingen™ antibody would not be a good candidate for the antibody-drug conjugate because it would not be specifically targeting the tumor associated Muc1, leading to potential toxicity side effects. It is likely that we are seeing this result because the BD Pharmingen™ antibody targets Muc1 on cells, regardless if it is glycosylated or not. In looking at the other two commercialized antibodies, while they are not binding to noncancerous cells, we see that there is only one or two cancerous cell lines that they are interacting significantly with.

In looking at the three antibodies that we have been synthesizing in the lab, we would expect that the antibodies show more specificity towards the cancer cells. This is because the antibodies were designed to specifically target the VNTR region, which is hypoglycosylated in tumor cells. HMFG2 is the one that stands out with its binding profile towards cancerous cells (H1975 and

MCF7), while only 2.17% of Muc1 expression on CCD19-Lu cells. The other two antibodies, SM3 and 12E, did not show as great of Muc1 expression on the cancerous cells. From this experiment, we decided that HMFG2 would be the best candidate as an antibody in an ADC to target tumor associated Muc1.

Anti-Muc1-DM1 Cytotoxicity

In the cytotoxicity assays, we were not able to obtain IC_{50} values for the mertansine (DM1) free drug. DM1 is a potent drug that disrupts the microtubule formation and depolymerizes already formed microtubules. Thus, this drug is most effective towards proliferative cells; these cells could be cancer cells or noncancerous cells. This quality is shown in Figures 10-14, which highlight that at the 24-hour time point, most of the cells are dead. Because of this, we were not able to obtain useful IC_{50} values.

One significant finding from the cytotoxicity assays was that the ADCs have a greater cytotoxic effect on Muc1 positive tumor cells than HMFG2 alone. From Table 4, we see that the HMFG2 antibody has an IC_{50} value of greater than 10 ug/mL for each cancer cell line. With the ADCs, HMFG2-DM1 R20 and R40, we see that there is a significant decrease in IC_{50} values. This shows that the ADC is more effective at killing Muc1 positive tumor cells than its parent antibody alone.

Another trend we see from Table 4 is that the ADC had little cytotoxic effect on the noncancerous CCD19-Lu cells. At all three timepoints, the ADC has an IC_{50} value of greater than 10 ug/mL. These findings are crucial because they show that our synthesized ADC is specifically targeting the hypoglycosylated Muc1 on the cancer cells, allowing for targeted cell killing.

From the cytotoxicity assay, we also noticed that with longer incubation times, the ADC exhibits more of a cytotoxic effect on the cancer cells. In Table 4, at the 72-hour time points, we are seeing anywhere from a 2 to 20 fold decrease in IC_{50} value compared to its respective 24-hour time point. We can hypothesize that with longer incubation times, the ADC is more effective because it has more time to become internalized within the cell, allowing for more time for drug release.

In comparing the A549 (lung adenocarcinoma) cell line to the other cancer cell lines, we see that the ADC is not exhibiting as large of a cytotoxic effect on these cells. It is likely we are seeing this effect because of the findings we found from the antibody binding assay, shown in Table 3. From this experiment, we showed that the A549 cells expressed that lower percentage of underglycosylated Muc1 detected by HMFG2 at 8.43%. With this finding, it is not surprising that the antibody did not show as great of a cytotoxic effect.

Mass Spectrometry

As to be expected, the drug-to-antibody ratios (DAR) that we obtained are larger for the ADCs that were synthesized at a larger molar ratio of drug to antibody. The DAR is an important characteristic of our ADC, especially when we are thinking about injecting this into a patient. With too many conjugated mertansine drugs to the HMFG2 antibody, we would likely see a decrease in solubility because of hydrophobicity. Further, this would cause the drug to become cleared from the system, resulting in it not reaching its target, thus decreasing its overall efficacy. As we move forward with potential *in-vivo* and clinical studies, we will have to maintain this balance.

FUTURE DIRECTIONS

In healthy tissues, Muc1 is a heterodimeric complex with the interaction between the Muc1-C and Muc1-N subunits [5]. It has been shown in recent studies that in cancer patients, there are elevated levels of Muc1-N in the serum due to its shedding from the Muc1-C subunit on the cell surface [34,35] (see Figure 19). In this state, Muc1-C functions as an oncoprotein through its interactions with many effectors shown to be linked to cancer progression, including the P13K-AKT and MEK-ERK pathways [36]. From its participation in these signaling pathways, Muc1-C has been linked to the induction of the epithelial-mesenchymal transition and epigenetic programming, contributing to cancer progression [36]. With our current ADC that targets the Muc1-N subunit, in thinking about clinical studies, its systematic delivery has to overcome the circulating Muc1-N in the serum in order for it to reach its target. We have recognized this as a potential complication and are currently in the process of generating an antibody that targets the Muc1-C subunit specifically.

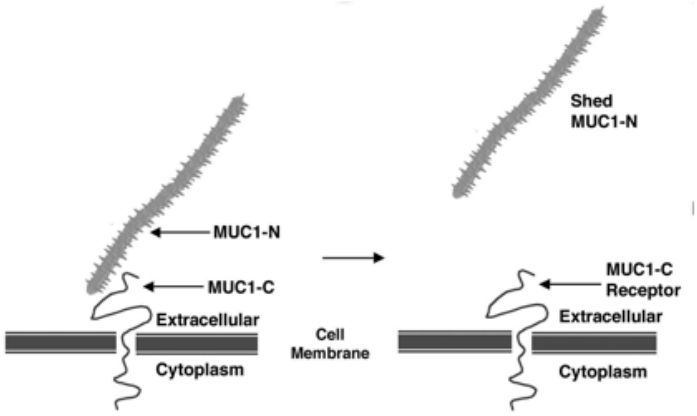

Figure 20: Interactions between Muc1-C and Muc1-N. In healthy tissue, Muc1 has been shown to be a heterodimer with the N and C subunits (left). In cancer-associated Muc1, it has been shown that the Muc1-N subunit is shed from the cell surface. Kufe (2009).

CONCLUSION

We have developed an antibody-drug conjugate that targets the hypoglycosylated Mucin1 seen in cancers like lung and breast (Specific Aim 1). We have also satisfied Specific Aim 2 by investigating the *in vitro* effects of the ADC compared to its parent antibody. The cytotoxicity assays have shown three significant properties of the synthesized ADC. First, the ADC has a greater cytotoxic effect on Muc1 expressing cancer cells compared to HMFG2 alone. Second, the ADC is specifically targeting the Muc1 associated with cancer cells, shown with its little effect on CCD19-Lu cells. Lastly, the ADC has a significant cytotoxic effect on Muc1 expressing cancer cells with longer incubation times. From our results, we have accepted our hypothesis of the combination of the anti-Muc1 antibody and cytotoxic agent produces an improved efficacy over antibody alone and reduced toxic side effects of drug alone, ultimately leading to the destruction of tumor-associated Muc1 cancer cells. In the future, we hope to compare these results to an ADC that targets the C-terminal region on Muc1. Our efforts lay groundwork for a novel treatment of cancers that overexpress Muc1.

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